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Full Length Research Paper

Growth assessments of *Nicotianatabaccum*cv.Xanthitransformed with *Arabidopsis thaliana*P5CS under salt stress

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Proline plays important role in osmo-adjusment and prevention of osmotic stress in plants. In order to analyze osmotic stress resistance, germination and growth pattern *in vitro* and *in vivo*, the *Arabidopsis thaliana*δ-1-pyrroline-5-carboxylate synthetase (P5CS) which is the regulatory enzyme of proline biosynthesis, was reverse transcribed, amplified into double stranded cDNA, cloned and transformed into tobacco using *Agrobacterium tumefaciens* mediated transformation. Seeds of chimeric T_0 transgenic plants were grown; T_1 and the succeeding T_2 transgenic plants were obtained. Subsequently, wild type and binary vector transformed control plants along with T_2 transgenic lines were exposed to salt concentrations from 0 to 250 mM during germination and growth stages. Results showed a significant difference ($P < 0.0001$) between T_2 and control plants, while the control plants germinated in the presence of salt concentrations up to 50 mM and tolerated 100 mM salt during growth phase. Transgenic T_2 lines were able to germinate in 200 mM NaCl and tolerated up to 250 mM salt during growth phase. Assessing proline contents, we observed 7.5 times more proline in T_2 transgenic plants than control plants in normal condition which increased up to 3.3 folds in stress condition. This osmotic stress resistance was further assessed for T_3 generation against drought and salt stresses *in vivo*. Results indicated osmotic stress resistance of T_3 generation as well as stable expression of P5CS along with achievement of homozygous transgenic lines.

Key words: δ-1-Pyrroline-5-carboxylate synthetase (P5CS), osmotic stress, transgenic plants, germination, growth phase.

INTRODUCTION

Drought and salinity are the two main limiting factors of plants growth and development. It is well known that application of selection programs that are effective in low stress conditions might lead to the selection of less tolerant cultivars (Perezde de vega, 1996). On the other hand, insensitive selection pressure could lead to the elimination of less adaptable genotypes, reduction of genetic variability and loss of extremes (Perezde de vega, 1996; Parsons, 1987). In this regard, expansion of the knowledge and application of the molecular mecha-nisms involved in plants response to biotic and abiotic stresses could help breeding and engineering plants being capable of coping against stresses. As a general response to osmotic stress, plants accumulate metabolites which confer them resistance against stress without interfering or affecting their metabolism (Orcutt et al., 2000). These compounds, which collectively are known as osmolytes are a wide range of molecules composed of sugars, sugar alcohols, methylated inositol, complex sugars, ions, positively charged metabolites and amino acids such as proline

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(Paul et al., 1996; Sun et al., 1999). Accumulations of osmolytes in the cytoplasm stabilize cellular osmotic pressure which in turn reduces accumulation of toxic ions, enhances enzymes stability against toxic ions such as Na^+, denaturing agents as well as osmotic stresses (Ashraf et al., 2007; Kishor et al., 2005; Okuma et al., 2000, 2002).

Among osmolytes, proline has shown to play a vital role against deleterious effects of ions and being involved in the stability of enzymes at higher temperatures (Banu et al., 2008; Arakawa et al., 1983; Arakawa et al., 1985; Schobert et al., 1978). Proline acts as a chemical chaperone conferring enzymes, conformational stability and activity (Ashraf et al., 2007; Okuma et al., 2000, 2002; Solomon et al., 1994). In addition, proline plays an important role in the adjustment of osmotic pressure of cells against stresses such as drought, salinity, low and high temperatures, insufficiency of nutrients, exposure to heavy metals, high acidity and ionizing radiation (Kishor et al., 2005). Parts of these activities could be attributed to the induction of the salt stress responsive genes, which possess proline responsive elements (PRE for example, ACTCAT) in their promoters (Chinnusamy et al., 2005).

The role of proline in osmo-adjustment has been reported in maize (Raya pati et al., 1991), “alfalfa” (Ginzberg et al., 1998) and *Arabidopsis thaliana* (Kiyosue et al., 1996). In maize, grown in low water potential, it was estimated that proline accumulation may account approximately 45% of the total osmotic adjustment in the root apex (Hasegawa et al., 2000; Votberg et al., 1991). Increased level of proline in response to osmotic stresses not only was observed in plants but also in a wide range of organisms such as bacteria, yeast, marine invertebrates and algae (Measues, 1975; McCue et al., 1990; Delauney et al., 1993).

Besides its stabilizing effects on cellular structure, proline is capable of detoxifying free radicals by forming long-lived adducts (Chen and Dickman, 2005; Chen et al., 2006; Floy et al., 1984; Okuma et al., 2004; Smirnoff et al., 1989). Hare and Cress (1997) hypothesized that, the $NADP^+$ / NADPH redox system may be important in metabolic regulation due to their involvement in the interconversion of the δ-1-pyrroline-5-carboxylate (P5C) to proline which could mediate the transfer of reducing equivalents from cytosolic NADPH to the electron transport (ET) chain. These inter-conversions could shuttle redox equivalents between cellular compartments, transfer of electrons from NADPH to $NADP^+$, coupling the oxidation of NADPH to mitochondrial ET and serving as a mechanism for energy production (Phang, 1985). Yeh and Phang (1988) demonstrated that, P5C enhances the activity of the oxidative pentose phosphate pathway.

Accumulation of proline in response to many commonly encountered stresses in the natural environments has prompted suggestion that genetic engineering and thus, production of higher levels of proline in crops might play a benefactor role against environmental stress (Hare et al., 1997). Under abiotic stress, accumulation of proline is thought to result from the induction of δ-1-pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-caboxylate reductase (P5CR) which are involved in the biosynthesis of proline from L-glutamate (Ashraf et al., 2007). Also, inhibition of enzymes involved in oxidation of proline such as prolinedehyrogenase (PRODH) and pyrroline-5-carboxylate dehydrogenase (P5CDH) would have positive impact on the proline accumulation (Delauney et al., 1993).

In *Arabidopsis* engineered with antisense proline dehydrogenase cDNA, the enzyme which is involved in the catabolism of proline, the results indicate accumulation of proline and constitutive tolerance to freezing temperatures as well as salinity (Nanjo et al., 2003). In accordance to mentioned findings, in the present study, *Nicotianatabacum* cv. Xanthi was transformed with the reverse transcribed P5CS cDNA of *A. thaliana*. The present report evaluates both morphological and physiological alterations in tobacco plants transformed with the *A. thaliana* P5CSin response to osmotic stress.

MATERIALS AND METHODS

Complementary DNA (cDNA) synthesis

Total RNA was extracted from *Arabidopsis* seedlings using guanidine hydrochloride method (Clark, 1997) and treated with RNase-free DNase I. First strand cDNA was synthesized (expand reverse transcriptase, Roche) and the oligo dT18 primer as per recommendations of the supplier. After dilution, 50 to 100 ng of the resulting cDNA was used as template for polymerase chain reaction (PCR) amplification using primers: Forward; 5'-TCAGTTAACTGGATCCTCTCTCTGTGTGTGGTTTT-3' and Reverse; 5'-GAAGAGAAGCGAATTCAATGTTCTTCATCGTACTT-3', specific for the P5CS cDNA with two mismatches in order to create restriction sites for enzymes, *Bam*HI and *Eco*RI (as underlined in primers sequences). The resulting product was cloned in *Bam*HI / *Eco*RI sites of the *plasmid*pBluescript SK^-(Stratagene) and transformed into *Escherichia coli* (DH5α) as a back up for P5CS for subsequent sub-cloning. Successful cloning was tested by applying restriction digestion, PCR amplification, dot blotting and sequencing.

Preparation of construct

In order to evaluate the effect of plant source of the original cDNA, P5CS from primary vector (pBluescript SK^-/P5CS) was excised with *Xho*I and *Bam*HI and used for subsequent subcloning and replacing the P5CS originated from *Vignaaconitifolia* (a kind gifted of Professor Verma, Ohio university, U.S.A. in pBI121 known as pBI

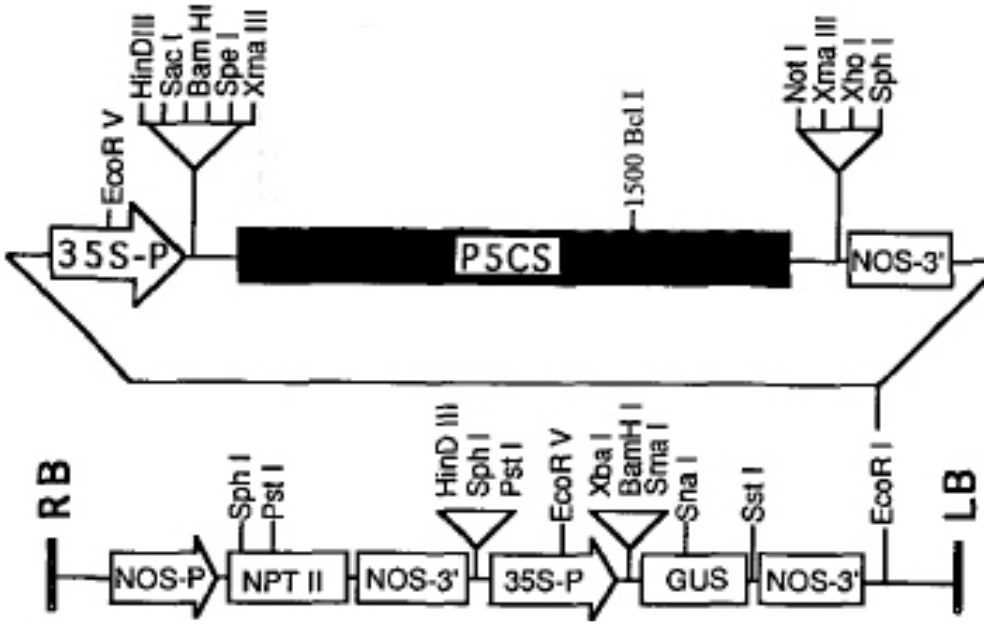


Figure 1. Vector used for expression of *Arabidopsis* P5CS cDNA in transgenic tobacco. The map of construct designed and used in *Agrobacterium* mediated tobacco plant transformation; pBI-P5CS X was constructed by replacing *Vigna* P5CS cDNA with double stranded cDNA of P5CS derived from *A. thaliana*. The entire cassette of the vector in both of constructs is the same.

P5CS (Kishor et al., 1995; Hong et al., 2000)) (Figure 1). The resultant P5CS was called pBI P5CS X, in which X stands for the applied *Xho*I restriction enzyme in the process of construction. This construct along with two other *Vigna* derived P5CS (Kishor et al., 1995; Hong et al., 2000) were used for *Agrobacterium* mediated tobacco plant transformation. All constructs were subsequently transformed into *E. coli* (strain DH5α) as back up and *Agrobacterium tumefaciens* for transformation.

Plant transformation and production of T_2 generation

Successful cloning, sub-cloning in vectors and transformation of plant were confirmed using restriction digestion, PCR, blotting, sequencing and reverse transcription polymerase chain reaction (RT-PCR). The *Arabidopsis* P5CS cDNA was sub-cloned into the binary vector; pBI121 as described earlier and used for plant transformation using *Agrobacterium* mediated transformation of tobacco leaf disks. Plants were also transformed with two *Vigna* P5CS cDNAs either with feedback inhibition or without inhibition as positive controls (Kishor et al., 1995; Hong et al., 2000). As an additional control (or control for the applied vector), pBI121 was also transformed to the plant leaf disks according to the earlier mentioned method. In addition, intact tobacco leaf disks were used as negative control. Following to callus formation, root and shoot forming plants were grown and after well adaptation of T_0 transgenic and control plants to the *in vivo* condition, they were transferred from *in vitro* culture medium to *in vivo* condition (pots containing vermiculite and composite; ½ : ½) and left for growth. Subsequently, plants were exposed to drought for confirmation of successful transformation in accordance with Yamchi et al. (2007). Transformed T_0 lines with high proline content were used for T_1 seed formation and plant production. T_1 seeds were cultivated in MS medium (Murashige et al., 1962) containing kanamycin (100 mg/l) in a controlled growth chamber (25 ± 2°C; cool white fluorescent light 200 µmol $m^{-2}s^{-1}$). Subsequently, plantlets were transferred into ½ vermiculite: ½ composite under earlier mentioned condition with 16 h of light and 8 h dark till grown well, proceeded self pollination and T_2 seed were produced.

Drought and salt treatment

T_2 generation of plants was treated with salt at both seed germination and growth phases. Twenty seeds of both T_2 and R_2 (wild type) obtained from the self pollinated T_1 and R_1 plants, were sown on MS-agar medium containing 0, 50, 100, 150, 200 and 250 mM NaCl. A 2 × 6 factorial experiment in a completely randomized design with 3 replicates was used. The experimental treatments included 2 plant types (T_2 transgenic seed versus R_2 wild type seed) at 6 levels of NaCl concentrations. After two weeks of sowing, percentage of germination was calculated on days 1, 3, 7 and 14. Analysis of variance on data was carried out using SAS version 6.12 software.

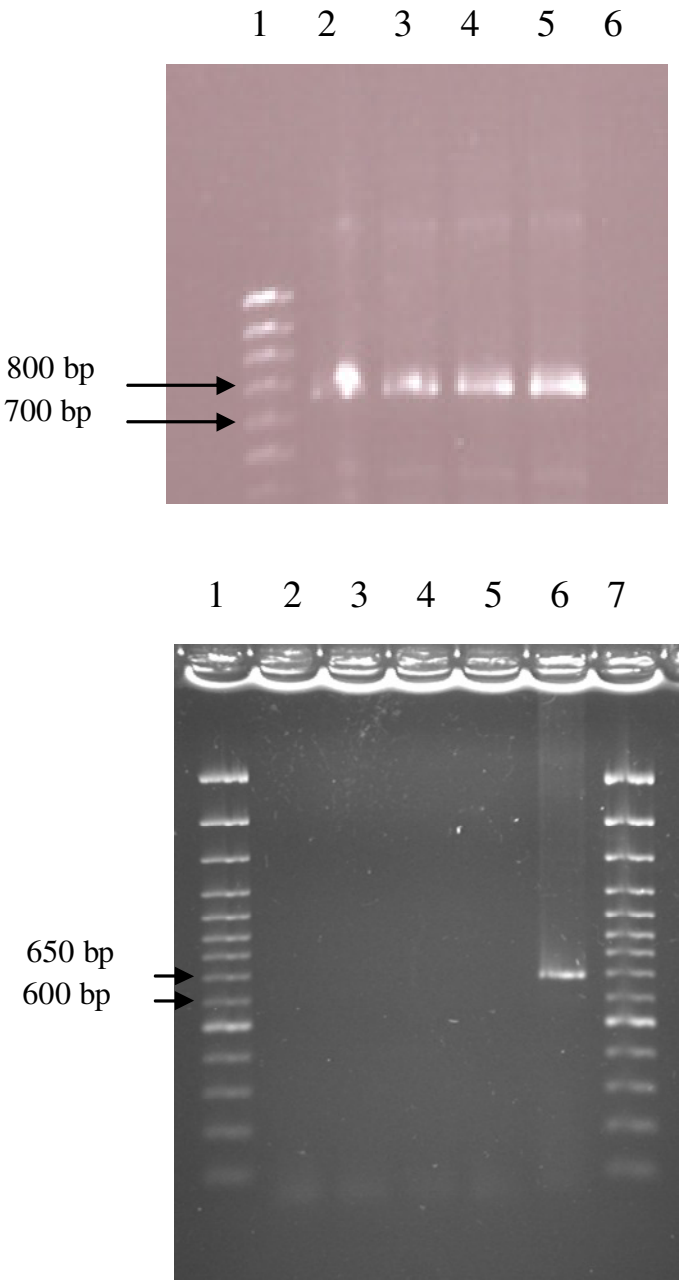


Figure 2. (A) Genomic PCR on DNA extracted from a pBI-P5CS X_9 transformed tobacco plant with primers designed such that upstream primer anneals inside 35S promoter and downstream primer within P5CS giving rise to 765 bp amplification product. Lane 1, Molecular size marker gene ruler 100 bp (Roche); lanes 2-5, PCR product of T_2 transgenic plant (pBI-P5CS X9), lane 6, PCR product of a non-transgenic plant; (B) RT-PCR of RNA isolated from P5CS transformed plant line resulting to amplification of 634 bpcDNA. Lane 1, Molecular size marker gene ruler 50 bp (MBI, Fermentas); lane 2, negative control; all PCR reagent plus water instead of T_2 transgenic cDNA (pBI-P5CS X_9), lane 3, wild type plant cDNA as negative control; lane 4, RT-PCR of the plant transformed with pBI121 as negative control; lane 5, further negative control where RNA instead of cDNA was used for checking the possible DNA contamination with the extracted RNA; lane 6, RT-PCR of T_2 transgenic cDNA (pBI- P5CS X_9); lane 7, molecular size marker gene ruler 50 bp (MBI, Fermentas).

Salt treatment of T_2 plants at growth phase was carried out by growing plants on 2 × 2 cm^2 plastic meshes placed on kanamycin-containing MS-agar medium. Three weeks later, plants were transferred into MS medium (suspension culture without agar with constant shaking for one month) containing 0, 50, 100, 200 and 250 mM NaCl. A 3 × 5 factorial experiment in a completely randomized design with 3 replicates was used. The experimental treatments were included 3 plant types (T_2 transgenic plant, R_2 wild type plant and plant transformed with pBI121) at 5 levels of NaCl concentration.

The plantlets were subsequently transferred and grown in pots as described earlier and seeds obtained from such plants were further cultivated in pots and used for evaluating drought and salt treatment of T_3 plants *in vivo* condition (Ferrario-Mery et al., 1998). For salt treatment, plants were exposed to ½ Hoagland's medium (Hoagland et al., 1938) containing 250 mM of NaCl for 21 days for salt stress and drought for 15 days.

Proline assay

Fresh and the uppermost well developed leaves (100 mg) of mature plant, were powdered in liquid nitrogen. Ten milliltres (10 ml) of 3% sulfosalicilic acid was added to each sample and centrifuged at 1300 rpm for 10 min. Two millitres (2 ml) of the supernatant from each sample and 2 ml of acidified ninhydrin solution (1.25 g ninhydrin dissolved in 30 ml acetic acid at boiling temperature to which 20 ml of ortho phosphoric acid was added) and 2 ml of 100% acetic acid were mixed and boiled for one hour. To stop further reaction, samples were immediately transferred onto ice-water for 20 min. To each samples, 4 ml of toluene was added at room temperature, mixed well and the absorbance at 520 nm was measured (Bates, 1973). For quantifying proline concentration, a calibration curve was prepared by measuring the absorbance of proline ranging in concentration from 1 to 160 µM.

Genomic DNA PCR and RT-PCR analysis

Based on the available cDNA sequence of the *A. thaliana* P5CS in NCBI (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/, Acc No; D32138) two sets of primers were designed and genomic DNA PCR was carried out. In order to avoid non specific annealing of primers with endogenous tobacco P5CS, the forward primer was designed from the internal sequence of 35S promoter. As a result, amplification was restricted to plants with integrated exogenous P5CS (Figure 2a). The first of the first set of the primers were; 5′ -GGATTGATGTGATATCTCCACTGACG-3' which anneals to the internal sequence of 35S promoter as forward primer and 5′-CCTTCAACATCGCTCAGAAGAATCAG-3' as the

reverse primer leading to a 765 bp PCR product from 5′ side of the P5CS. In addition, for limiting PCR amplification only to the cloned P5CS, the forward primers were designed to start few nucleotides before the end of one exon and the start of the next exon. Accordingly, amplification was restricted only to the reverse transcribed cDNA of the cloned P5CS. The sequence of the second set of primers was the forward; 5′-CCAAGGGCAAGTAAGATACTGAACAT-3′ and the reverse primer; 5′-GCAAGACTAAGTGGTAAAGTGGATCT-3′, leading to 634 bp amplification product from 3' side of the P5CS. PCR mixture was composed of 1 µl DNA (25 to 50 ng), 10 pmole of each primer, 200 µM of each dNTP, 2 mM $MgCl_2$, 1X PCR buffer and 1U *Taq* DNA polymerase (Roche) in a 25 µl final volume. PCR started with initial denaturation at 95°C for 5 min and followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min. Final extension was done at 72°C for 5 min. Post amplification product was electrophoresed in 1.2% agarose/tris-borate-ethylenediaminetetraacetic acid (*TBE*) gel at 80 V for 45 min and visualized with ethidium bromide staining.

In order to check transcription status of P5CS in transgenic, plant RT-PCR was carried out. Total RNA from transformants was isolated using RNeasyQiagen plant mini kit (Qiagen) and treated with RNase-free DNase I in order to validate the expression of exogenous P5CS and suspended in 30 µl of RNase-free water. cDNA was synthesized using M-MuLv reverse transcriptase (MBI, Fermentas). The RT-PCR was carried out using specific primer pairs for P5CS cDNA, such that, forward primer was composed of 5'-CCAAGGGCAAGTAAGATACTGAACAT-3' and the reverse primer 5'-GCAAGACTAAGTGGTAAAGTGGATCT -3', respectively. The amplification conditions were the same as that for genomic PCR and aliquot of each of the reactions were run on 1.2% agarose/TBE gel.

Southern blotting

Genomic DNA was isolated from young leaves of transgenic and wild type plants (Murray et al., 1980) and 30 µg of which were used along with 2 µg of each of the purified pBI-P5CS X plasmids as positive control and pBI121 plasmid as negative control in digestion reaction with *Sac* I and *Bcl* I restriction enzymes according to the restriction map of *Arabidopsis* P5CS cDNA. Digested DNA were resolved on a 0.7% agarose gel, blotted to nylon membrane (Roche), baked at 80°Cfor 2 h and prehybridized in a buffer containing 5X saline sodium citrate (SSC) (0.075 M NaCitrat, 0.75 M NaCl, pH7.0), 0.1% N-lauroylsarcosine, 0.02% SDS and 1% blocking reagent. Subsequently, overnight exposure to the denatured DIG labeled probe (*Arabidopsis* P5CS cDNA was used as a probe) in blocking reagent at 68°C was carried out. Membrane was washed with 2X SSC buffer containing 0.1% SDS followed by 0.1X SSC buffer containing 0.1% SDS. Anti- *digoxigenin* conjugated alkaline phosphatase was applied, membrane was washed and substrate CSPD (di-sodium 3-(4-metho-xyspiro (1,2-dioxetane-3,2'-(5'-chloro)tricycle(3.3.1.1^{3,7}) decan)-4-yl) phenyl phosphate in detection buffer (0.1 M Tris-base, 0.1 M NaCl, 50 mM $MgCl_2$ pH 9.5) was added. Subsequently, the membrane was exposed to the x-ray film and kept in the dark till image development (Sambrook et al., 2001).

GUS assay

The GUS assay was performed according to Gallagher (1990). Leaf discs from control and transgenic plants were prepared and completely immersed in X-Gluc solution (0.1 M sodium phosphate buffer, pH 7.0; 10 mM EDTA; 0.5 mM potassizumferricyanide, pH 7.0; 1.0 mM X-glucuronide and 1% Triton X 100). Incubation was carried out at 37°C for at least 10 min or longer until formation of complete bluish-green color.

Peptide synthesis and production of anti *Arabidopsis* P5CS

The complete amino acid sequence of *A. thaliana*, ecotype; Columbia) was obtained from NCBI (Acc no; D32138, http://www.ncbi.nlm.nih.gov/) and 15 amino acids from N-terminal (EELDRSRAFARDVKR) was chosen for raising antibody against P5CS according to Nanjo et al. (1999). Polypeptide was synthesized (Sigma-Genosys) and the primary immunization was carried out by sub-coetaneous injection of 200 µg of polypeptide in complete Freund's adjuvant into 4 sites on rabbit back near the lymph nodes. Further boosting was done three times (intra-muscular) each at 4 weeks intervals with 200 µg of polypeptide prepared in the incomplete Freund's adjuvant (BioGene). After heart blood collection at the end of immunization, serum was collected and used for protein detection at 1/1000 dilution.

Immunoblotting

For immunoblotting, 200 mg fresh leaves from T_2 transgenic and control plants were powdered in liquid nitrogen, transferred into 1.5 ml of homogenizing buffer containing 10 mMTris-Cl pH 7.4; 5 mM $MgCl_2$; 1 mM PMSF and was incubated in ice for 20 min. Subsequently, SDS (2%) and β-mercaptoethanol (5%, v/v) was added. Following centrifugation at 13000 rpm for 20 min at 4°C, supernatant was transferred to new vial and was used in Bradford protein assay. Total soluble proteins (50 µg)

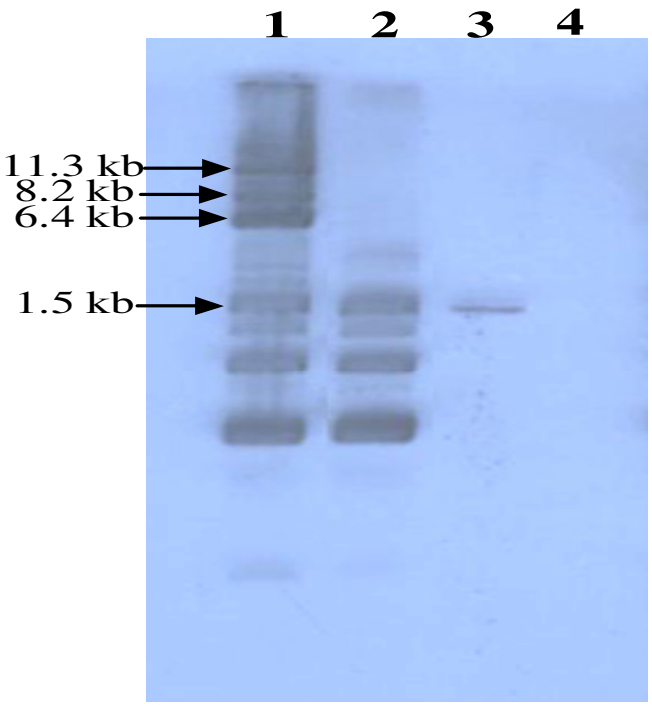


Figure 3. Southern blot analysis of T2 transgenic plant obtained from transformation with pBI-P5CS X. Lane 1, T_2 transgenic plant (pBI-P5CS X_9); lane 2, non-transgenic plant (wild type plant); lane 3, purified pBI-P5CS X9 plasmid; Lane 4, pBI121 as a negative control.

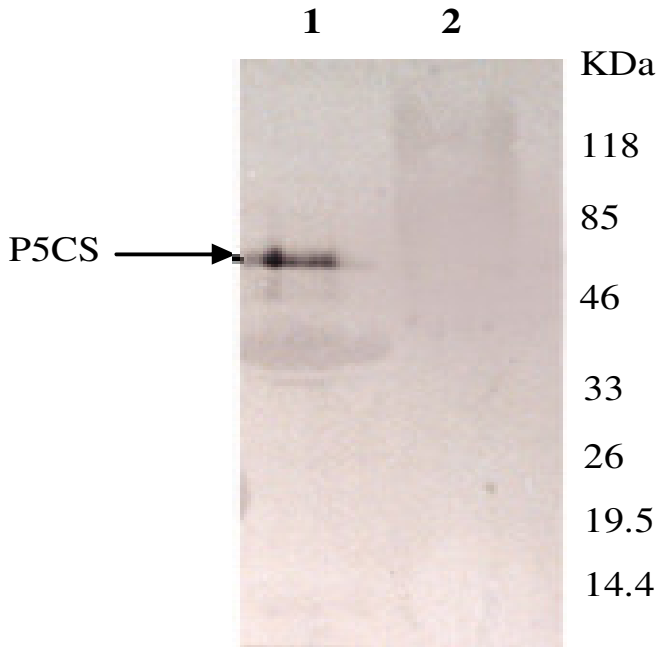


Figure 4. Immunoblotting of the transformed δ-1-pyrroline-5-carboxylate synthetase by applying antibody raised against *Arabidopsis* P5CS. Lane 1, T_2 transgenic plant (pBI- P5CS X_9); lane 2, non-transgenic plant (wild type plant).

were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and immunoblotted, using antibodies raised against the purified *Arabidopsis* P5CS protein.

RESULTS AND DISCUSSION

Expression of *Arabidopsis* P5CS cDNA in transgenic tobacco plants

Following the reverse transcription and amplification, *Arabidopsis* P5CS was cloned in Pbluescript SK^- andsubsequently subcloned in pBI121 by replacing *Vigna* P5CS (Figure 1). This clone along with two other P5CSs obtained from *Vigna* (pBI-P5CSs with and without feedback inhibition) was used for subsequent analysis. As it is well known, 35S promoter was originally derived from cauliflower mosaic virus, a *Brassicaceae* specific infecting virus. Thus, we hypothesized that 35S promoter may also promote *Arabidopsis* P5CS as efficient. Formerly, Kishor et al. (1995) have reported transformation of tobacco plant with the *Vigna* P5CS cDNA under 35S promoter control. In the present study, the same vector was used except that *Vigna* P5CS was substituted with *Arabidopsis* P5CS and then transformed into tobacco.

Molecular characterization of transgenic tobacco plants

In order to verify that the gene of interest was successfully cloned, expressed and translated, molecular analysis on the T_2 transgenic plants were carried out at three levels including DNA amplification, southern blotting along with RT-PCR and immunoblotting. Results (Figure 3) showed that, all samples including the wild type tobacco were reacted with the probe, indicating that *Vigna* P5CS cDNA not only hybridizes with *Arabidopsis* P5CS (pBI-P5CS X, either as an isolated vector or integrated into tobacco genome), but also with putative endogenous tobacco P5CS.According to this figure, three copies of P5CS cDNA were integrated with the genome of the plants. Based on *Arabidopsis* P5CS cDNA restriction map (Figure 1), *Sac* I and *Bcl* I restriction enzymes liberate a 1500 bp fragment from 5' terminus of the P5CS cDNA (Figure 3; lane 3). This fragment was detected in all transgenic tobacco plants. In addition, three bands with different sizes were also detected in T_2 transgenic plants (indicated with arrows), suggesting the insertion of three repeats of transfer DNA. The shorter bands in both transformed and non-transformed plants (Figure 3, lanes 1, 2) represent the endogenous genomic tobacco *P5CS*.

To verify whether exogenous P5CS is expressed and translated in transformed plants, immunoblotting was done using polyclonal antibody raised against *Arabidopsis* P5CS in rabbits. Figure 4 indicates that, the expression of *P5CS* is limited to the transformed plants. While expres-

Table 1. Proline content of fresh leaves from control and P5CS transgenic T_0 plants grown under normal and after five days of drought stress conditions (standard errors in parenthesis).

Plant	Proline content (µg g-1) under well watered condition	Proline content (µg g-1) after five days of drought stress
Non-transformed plant (control)	8.90 (2.1)	12.53 (3.2)
Transformed plant (pBI P5CS X5)	150.91 (11.3)	372.52 (20.4)
Transformed plant (pBI P5CS X6)	200.27 (13.5)	204.45 (13.9)
Transformed plant (pBI P5CS X7)	282.48 (18.1)	483.05 (26.3)
Transformed plant (pBI P5CS X8)	270.23 (17.6)	490.18 (25.7)
Transformed plant (pBI P5CS X9)	265.87 (17.3)	520.25 (26.5)

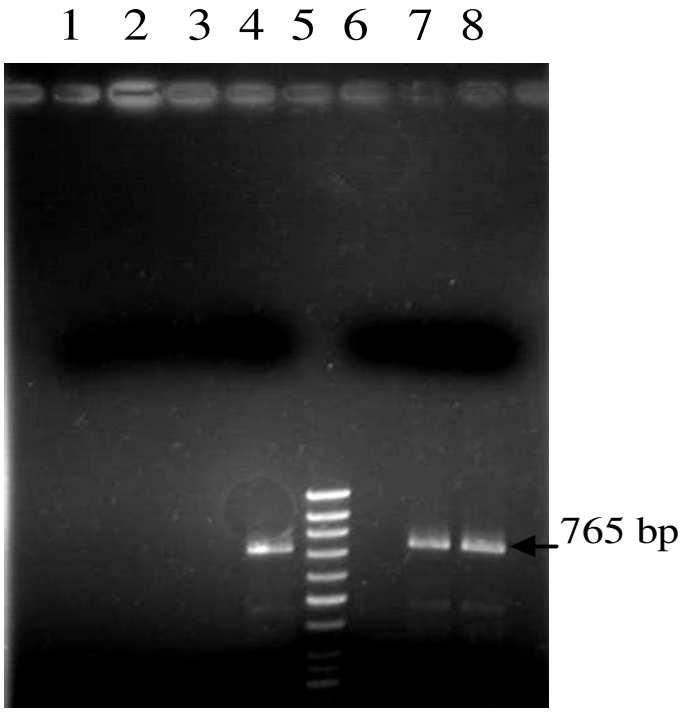


Figure 5. Genomic PCR on the DNA extracted from a pBI-P5CS X_9 transformed tobacco plant (T_0 plant) with primers designed such that the upstream primer anneals within 35S promoter and the downstream primer within internal P5CS sequence, giving rise to 765 bp amplification product. lane 1, Negative control; All PCR reagent plus water instead of DNA; lane 2, PCR product of a non-transgenic plant; lane 3, PCR product of plant transformed with pBI121; lane 4, PCR product of T_0 transgenic plant (pBI-P5CS X_9); lane 5, molecular size marker gene ruler 100 bp (MBI, Fermentas); lanes 6 and 7, repeat of PCR on the DNA extracted from T_0 transgenic plant (pBI-P5CS X_9). A negative result in lane 6 could be interpreted as a result of mosaic phenomenon in the transformation; lane 8, PCR product on the plasmid contain *Arabidopsis* P5CS cDNA as positive control.

sion of P5CS in non-transgenic plant in normal (non-stress) condition could not be detected, the expression of P5CS in transgenic plant under the same condition was relatively high and detectable as a 77-kDa band at the expected size in western blot analysis.

Assessment of T_0 transgenic plants proline content and generation of T_1 and T_2 transgenic plants

Results obtained in this part of study indicated that, proline content of transgenic plant in non-stress and stress conditions was much higher than control plants (Table 1). The higher proline content of transgenic plants under well watered condition could be attributed to the basal 35S promoter activity. On the other hand, following to five days of drought, no significant increase in proline content was detected in the control plant which is in contrast to the previous report. This difference in results might be explained to be due to the duration of applied stress. On the other hand, following to this period of stress, proline content in transgenic plants (except pBI P5CS X6) was increased two to three fold (Table 1), which is in agreement with the previous observations (Kishor et al., 2005a). To investigate expression status of the exogenous P5CS in T_0 plants, DNA and RNA were extracted from leaves of transgenic X9 plant and PCR or RT-PCR was carried out under the conditions explained earlier. Interestingly, it was found that not all cells were transformed with exogenous P5CS (Figure 5). Leaves with negative PCR response were also negative for GUS assay (data not shown). Also, as Table 1 points out, among transgenic plants, proline content of leaves were significantly different ($p<0.01$). This inconsistency within transgenic T_0 plants may be caused by the heterogeneity in terms of P5CS expression. Since transgenic plants were generated by shooting from callus, it was possible that both transformed and non-transformed cells were present in the same shoot. Non-transformed cells could grow and reproduce probably because of degradation of kanamycin in the medium by neighboring transformed cells. Such phenomenon was also previously reported by other groups (Sherman et al., 1998).

Verifying P5CS expression pattern in transgenic plant

Under normal non-stress condition, the leaves of T_2 generation of pBI P5CS X_9 contained 1194 µg proline per gram fresh leaves, a value not significantly different (p-

Table 2a. Percent of germination of T_2 generation transgenic and wild type plants under normal and salt-stress conditions (standard errors in parenthesis).

Salt concentration (mM)	Wild type	Transgenic (pBI-P5CS X_9)
0	0.69[a] (0.006)	0.70[a] (0.006)
50	0.25[d] (0.008)	0.36[b] (0.014)
100	0 [f]	0.30[c] (0.002)
150	0 [f]	0.19[e] (0.026)
200	0 [f]	0.18[e] (0.009)
250	0 [f]	0 [f]

Means with non-overlapping sets of alphabets differ significantly from each other.

Table 2b. Analysis of variance for percent germination of T_2 generation transgenic and wild type plants on day sixteen under normal and salt-stress condition.

Source	Df	ANOVA SS	Mean square	F value	Pr > F
Plant	1	0.01000	0.01000	30.77	0.0001
Dose	5	0.04970	0.00994	30.58	0.0001
Plant * Dose	5	0.01110	0.00222	6.83	0.0004
Error	24	0.00078	0.00032		
Corrected total	35	0.07860			

R-square	C.V.	Roots MSE	Proline mean
0.90076	1.988	0.018027	0.2225

DF, Degree of freedom.

value) from former published data (1000 µg per gram fresh leaf (Kishor et al., 1995) (Table 2). Low levels of proline content have also been observed in other plants in normal condition (Hmida-Sayari et al., 2005). They estimated 200 proline µg per gram of fresh leaves of P5CS transformed potato plant. Increasing salt concentration up to 200 mM in our study has resulted to enhanced proline content up to 2938 µg per gram fresh leaves which again was comparable to the results shown elsewhere (2600 µg per gram fresh leaf; (Table 2) for tobacco and 1126 µg per gram fresh leaves for potato in response to 180 mM salt (Hmida-Sayari et al., 2005). Based on these results, it could be concluded that 35S promoter may promote expression of both types of P5CS genes (*Vigna* versus *Arabidopsis*) to the same extent regardless of the origin of the genes or their natural relationship to the promoter. It is well known that 35S is a strong promoter (Hammond et al., 2000), but whether the stated suggestion could be extended to weak or inducible promoters remains to be explored.

Expression of P5CS, germination and growth of plants under salt stress

Using PCR, we found that cells from T_2 plants were homozygous for exogenous P5CS and stably expressed the transformed*Arabidopsis* P5CS cDNA. Following the evaluation of T_2 plants for salt tolerance, seeds from these plants were used for evaluating growth and germnation pattern under normal and stress conditions. Results obtained on days 1, 3, 7 and 16 after onset of germination was recorded in four different tables for analysis of variance. There was no significant difference between control and transgenic plants for germination on day one. However, the differences were significant ($P < 0.0001$) for days 3, 7 and 16 (Table 3a, b; Figure 6b), similar to the reports of Gelvin et al. (1989) on rice germination or Zhang et al. (2010) on barley germination and indicated a gradual increase in proline content of plants during the period of stress. Results showed that, control plants and plants transformed with vector (pBI121) were both similarly able to tolerate up to 100 mM NaCl in growth (Figures 7 and 8; Table 2a) and up to 50 mM NaCl in germination phases, respectively (Figure 6a, b; Table 3a). In contrast, transgenic plants were tolerant up to 250 mM NaCl in growth and 200 mM in germination phases, respectively (Figures 6 and 7; Tables 2a and 3a). Therefore, it was concluded that both transgenic and non-transgenic plants were more sensitive to salt stress during germination phase than to the growth phase.

A comparison of proline content of control and pBI121 transformed plants from 0 mM up to 100 mM of NaCl, revealed no significant differences (Figure 7; Table 2a). However, transgenic plants showed a significant difference in proline content not only for this range of salt

Table 3a. Least-square estimates of proline contents (µg/g *in vitro* fresh leaves) in T_2 generation transgenic plants, tobacco transformed with pBI121 and wild type plants under normal and salt-stress conditions (standard errors in parenthesis).

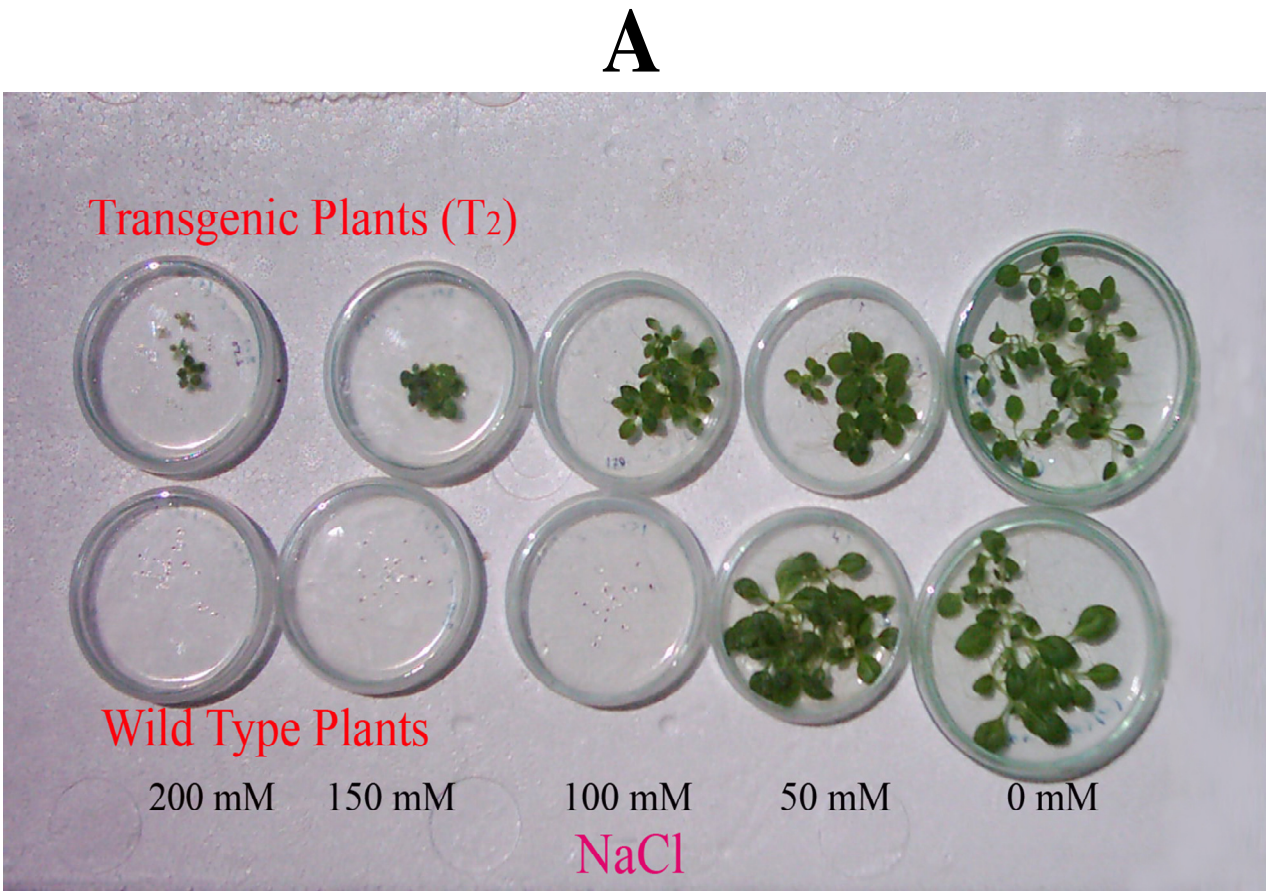
Salt concentration (mM)	Wild type	pBI121	Transgenic (pBI-P5CS X_9)
0	158.48^{ef} (7.779)	165.53^{ef} (7.570)	1194.08^{d} (29.034)
100	229.06^{ef} (8.883)	367.50^{f} (19.155)	2088.54^{c} (32.855)
200	Lost	Lost	2937.93^{b} (108.773)
250	Lost	Lost	3835.94^{a} (144.741)

Means with non-overlapping sets of alphabets differ significantly from each other.

Table 3b. Analysis of variance for proline contents (µg/g *in vitro* fresh leaves) in T_2 generation of transgenic plants, tobacco transformed with pBI121 and wild type plants under normal and salt-stress conditions.

Source	Df	ANOVA SS	Mean square	F value	Pr > F
Plant	2	46051513.68101	23025756.840508	2611.49	0.0001
Dose	3	2736266.567963	912088.855987	103.45	0.0001
Plant * Dose	6	9209483.477761	1534913.912960	174.08	0.0001
Error	24	211609.8881333	8817.078672		
Corrected total	35	58208873.61487			

R-square	C.V.	Roots MSE	Proline mean
0.996365	10.26496	93.8993007	914.7558333



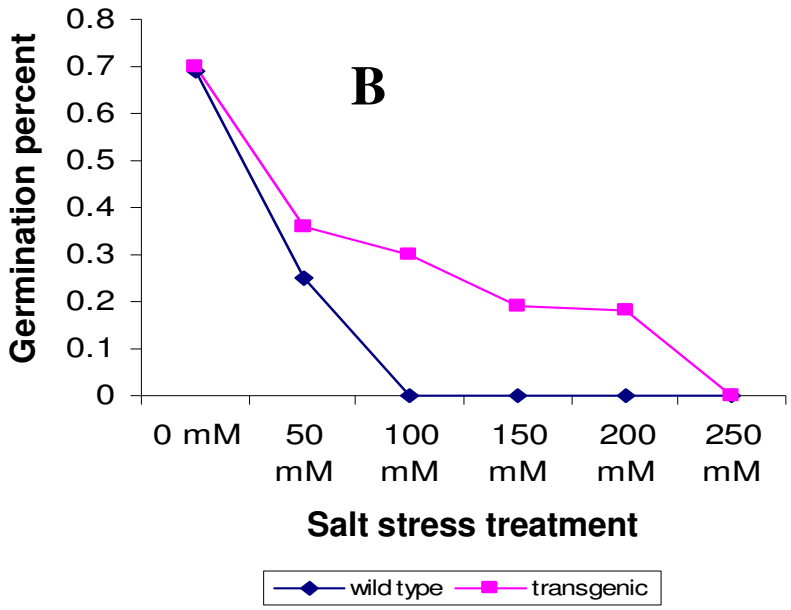


Figure 6.(A and B) Germination profile and percent of T_2 generation transgenic versus wild type seeds under normal and salt-stress condition.

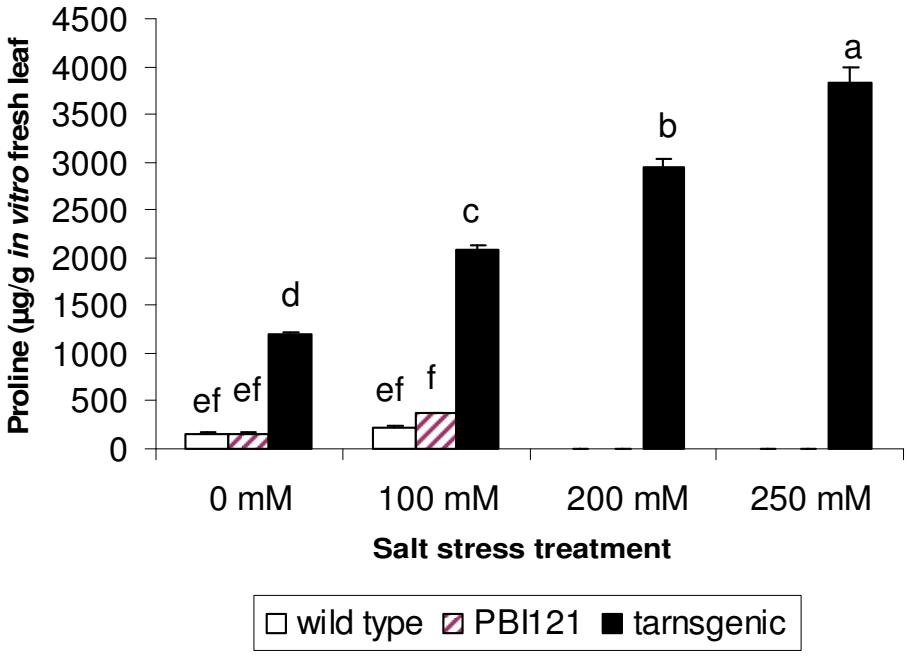


Figure 7. Histogram of proline assay on T_2 generation transgenic and wild type plants under normal and salt stress conditions. Means with non-overlapping sets of alphabets differ significantly from each other. No growth could be observed for wild type and pBI 121 transformed plants in 200 and 250 mM of salt concentration.

concentration but also from 100 to 250 mM of salt where control plants were no more tolerant (Figure 7).

Figure 9a shows the response of control and transgenic plants to two weeks of drought stress and Figure 9b to 250 mM of salt for three weeks. As Figure 9a indicates, while control wild type plants display sensitivity to the drought as could be observed by the higher degree of wilting, transgenic plants are more resistant. On the other

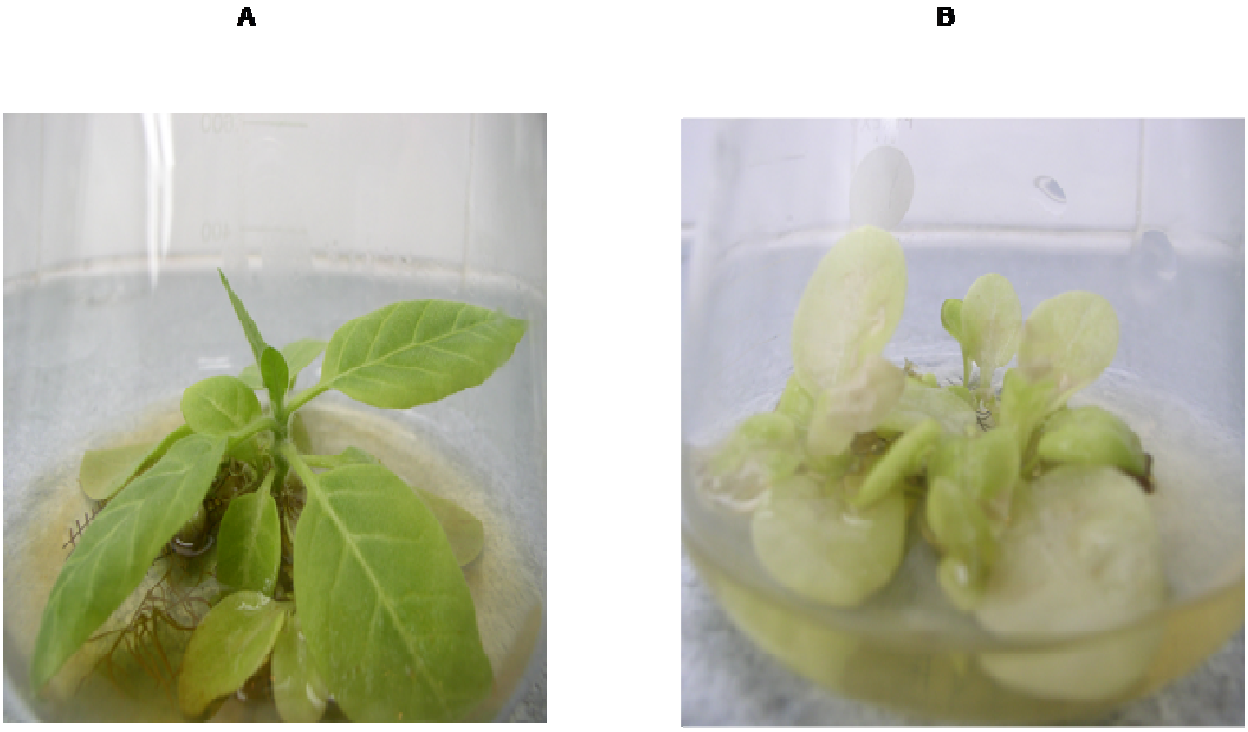


Figure 8. Suspension cultures of T_2 transgenic (pBI-P5CS X_9) (A) and wild type plants (B) on MS media containing 250 mM NaCl.

Figure 9. Growth pattern of T_3 transgenic versus control plants exposed to 15 days of drought (A); or 21 days of exposure to 250 mM salt (B).

hand, up on application of salt stress we observed retardation in the growth and more deformation of leaves in the wild type plants.

Under normal condition, the proline content of transgenic plants was 7.5 times more than that of control plants (1194.04 µg of proline per gram fresh weight of P5CS transformed plants versus 165.53 µg of proline per gram fresh weightof controlplants). This findingwas inaccordance with Kishor et al. (1995), who reported 1000 µg proline per gram fresh weight of transformed plants and 100 µg per gram fresh weight of control plants. How-ever, our results are not in agreement with the report by Yamada et al. (2005), who observed 1.3 to 3.5 folds of proline contents in Petunia with respect to the osmotic stress. On the other hand, transgenic plants responded to the salt stress by 2.5 to 3.3 folds further increase in proline content compared with the same plants under normal non-stress condition.

The amount of proline was measured to be 2938 up to 3836 µg per gram fresh weight of transformed plants in salt stress compared with the 1194 µg of proline per gram fresh weight in non-stress condition.

In addition to the *Arabidopsis* P5CS cDNA, two other *Vigna* P5CS vectors were used as positive controls. Among these two vectors, one displayed functional feedback inhibition by proline, whereas the other is not responsive as a result of disrupted feedback inhibition following to site directed mutagenesis (Kishor et al., 1995; Hong et al., 2000). Comparing salt tolerance for all three types of transformed plants (plants transformed with *Arabidopsis* P5CS cDNA and plants transformed with *Vigna* P5CS cDNAs; either with or without feedback inhibition) we observed the uppermost tolerance up to 250 mM of salt. From these observations, it was concluded that increase in proline level could confer plants withstanding up to specific concentration of salt. One cause for the observed similarity could be partly, due to 35S promoter activity that drives expression of both types of P5CSs. The other cause could be structural and functional similarities between two types of P5CSs that demands further future investigations.

In conclusion, our study indicates achievement of transformed plants that stably expressed P5CS up to three generations. Our further analysis on the fourth generation also supports this stability (data not shown). Comparing to the wild type plants, the transformed plants are tolerant up to four folds of salt concentrations. The successful maintenance of P5CS expression for several generations in model plants with successful transformation could be helpful in obtaining crops tolerant against osmotic stresses.

Abbreviations:P5CS, δ-1-Pyrroline-5-carboxylate synthetase; **NADPH,** nicotinamide adenine dinucleotide phosphate; **P5CR,** δ-1-pyrroline-5-carboxylate reductase; **PRODH,** proline dehyrogenase; **P5CDH,** pyrroline-5-carboxylate dehydrogenase; **PCR,** polymerase chain reaction; **RT-PCR,** reverse transcription polymerase chain reaction; **TBE,** tris-borate-ethylenediaminetetraacetic acid; **SSC,** saline sodium citrate; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **EDTA,** ethylenediaminetetraacetic acid.

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